

## PHYSIOLOGICAL RESPONSES OF THE CRAYFISH *PACIFASTACUS LENIUSCULUS* TO ENVIRONMENTAL HYPEROXIA

### I. EXTRACELLULAR ACID–BASE AND ELECTROLYTE STATUS AND TRANSBRANCHIAL EXCHANGE

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#### Summary

Extracellular acid–base and ionic status, and transbranchial exchange of acidic equivalents and electrolytes, were monitored in freshwater crayfish (*Pacifastacus leniusculus*) during control normoxia ( $P_{O_2} = 148$  mmHg; 1 mmHg = 133.3 Pa), 72 h of hyperoxia ( $P_{O_2} = 500$  mmHg) and 24 h of recovery. An initial (3 h) respiratory acidosis of 0.2 pH units was completely compensated within 48 h by a 50% increase in metabolic [ $HCO_3^- + CO_3^{2-}$ ] accompanied by a significant reduction in circulating [ $Cl^-$ ]. In addition, the original increase in  $P_{CO_2}$  was partially accommodated. The time course of transbranchial acidic equivalent exchange paralleled the change in extracellular metabolic base load with a significant branchial output of  $H^+$  during the first 48 h of hyperoxia. This was associated with net branchial effluxes of  $Cl^-$  and  $Mg^{2+}$ . Unidirectional flux analysis revealed parallel reductions in  $Na^+$  influx and efflux during initial hyperoxic exposure, reflecting an alteration in exchange diffusion. The net  $Cl^-$  efflux was due to an initial increase in efflux followed by a reduction in influx. The reverse sequence of events occurred more rapidly when normoxia was reinstated: metabolic base was removed from the haemolymph and control haemolymph acid–base and ion levels were re-established within 24 h. Transbranchial fluxes of acidic equivalents similarly recovered within 24 h although net  $Na^+$  output and  $Cl^-$  uptake persisted.

The study attempted to identify relationships between branchial net  $H^+$  exchange and components of  $Na^+$  and  $Cl^-$  exchange and quantitatively to correlate changes in the acidic equivalent and electrolyte concentrations in the extracellular fluid compartment with those in the external water.

#### Introduction

Aquatic animals regulate extracellular pH primarily by transepithelial transfer of acidic/basic equivalents (i.e.  $H^+$ ,  $NH_4^+$ ,  $HCO_3^-$  or  $OH^-$ ). Hyperventilation

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on account of the low  $O_2$  content of water renders  $P_{CO_2}$  levels relatively insensitive to changes in ventilation.

Recent studies of fish (Wood *et al.* 1984; Heisler, 1986) have adopted an integrated approach to acid–base homeostasis by examining a number of body fluids, including the extracorporeal water compartment, and assessing the relative roles of available exchange epithelia. A similar approach was used in the present study on the freshwater crayfish. The decapod crustaceans differ from lower vertebrates in a number of physiological respects which could have a bearing on acid–base regulation. First, the extracellular fluid, which comprises a single functional compartment, occupies 30% of body mass compared to 5% in fish where blood contains two compartments (i.e. erythrocytes and plasma). Second, the skeletal matrix composed of carbonate and bicarbonate is located externally and undergoes a cyclical process of mineralization recognized as moulting. Continuous deposition and resorption processes occur in the vertebrate endoskeleton.

In the present study, experimental hyperoxia was used to depress ventilation and induce extracellular acidosis (Truchot, 1975; Sinha & Dejours, 1980). Hypercapnia has been used in the past for this purpose (Cameron, 1978) although  $CO_2$  is less important than  $O_2$  in setting ventilatory drive in water breathers. Varying degrees of metabolic compensation were reported during hyperoxic exposure of the European crayfish *Astacus* (Dejours & Beekenkamp, 1977; Dejours & Armand, 1980; Gaillard & Malan, 1983).

The ion uptake mechanism on the gills of the freshwater crayfish appear to exchange  $Na^+$  for  $H^+$  or  $NH_4^+$  and  $Cl^-$  for  $OH^-$  or  $HCO_3^-$  (Krogh, 1939; Shaw, 1959, 1960*a,b*; Bryan, 1960; Kirschner *et al.* 1973; Ehrenfeld, 1974). However, no one has attempted to determine whether these electroneutral ion exchanges are employed in acid–base regulation as they are in fish (Wood *et al.* 1984).

This paper is the first in a series of four which attempt to delineate the mechanisms of acid–base regulation during hyperoxia in the crayfish *Pacifastacus leniusculus*. In the present study, changes in extracellular acid–base and ionic status are correlated with transbranchial fluxes of acidic equivalents and electrolytes. Subsequent papers address the role of the antennal gland which is the functional analogue of the kidney (Wheatly & Toop, 1989), identify corresponding changes in intracellular acid–base status (M. G. Wheatly, R. Morrison, T. Toop & L. C. Yow, in preparation) and identify transmembrane exchanges in different tissues including the carapace (M. G. Wheatly & E. C. Vevera, in preparation).

## Materials and methods

### *Experimental animals*

Adult intermoult crayfish *Pacifastacus leniusculus leniusculus* (Dana) of mean mass  $26.0 \pm 1.7$  g ( $N = 16$ ) were obtained from Pacific Crayfish Co. in California. For at least 2 weeks prior to experimentation, they were housed, in groups of 20 in 30-l aquaria, in aerated Gainesville tap water ( $12^\circ C$ ; 12 h: 12 h light: dark) with the

following ionic composition (in mequivl<sup>-1</sup>): Na<sup>+</sup>, 0.55; K<sup>+</sup>, 0.04; Ca<sup>2+</sup>, 1.15; Mg<sup>2+</sup>, 0.85; Cl<sup>-</sup>, 0.73; titration alkalinity, 1.80; pH 7.8. The water was recycled through a bottom filter and polyvinylchloride pipes were provided as refuges. Seven days prior to an experiment, crayfish were surgically prepared for postbranchial haemolymph sampling as outlined by Wheatly & McMahon (1982). Crayfish were not fed for 1 week prior to experimentation to minimize any influence of feeding on ion or acid–base exchange (Wood & Caldwell, 1978).

#### *Experimental protocol*

The complete study involved six series of experiments. Findings from the first two are reported in this paper (see Wheatly & Toop, 1989, for series 3 and 4; M. G. Wheatly, R. Morrison, T. Toop & L. C. Yow, in preparation, for series 5 and 6; M. G. Wheatly & E. C. Vevera, in preparation, for series 6). Prior to the start of an experiment, eight crayfish were acclimated for 48 h in individual rectangular chambers (12 cm × 6.5 cm × 5.5 cm) in 250 ml of tap water at constant temperature (12°C). At the base of each container was a perforated circular tube which delivered a continuous stream of humidified air providing a normoxic oxygen tension (P<sub>O<sub>2</sub></sub>) of around 148 mmHg which was measured continuously on a thermoequilibrated IL O<sub>2</sub> electrode (20984) connected to an IL 213 blood gas analyser. The experimental water was replaced every 12 h with minimal disturbance to the animal. At the commencement of an experiment (t = 0 h), the gas supply was switched from humidified air to humidified O<sub>2</sub>, increasing P<sub>O<sub>2</sub></sub> within 2 min to approximately 500 mmHg. Hyperoxia was maintained for 72 h, after which normoxia was re-established for 24 h to study recovery processes.

Series 1 reports time-dependent changes in haemolymph acid–base and electrolyte concentrations in the control period (t = -24 h), and during hyperoxia (t = 3, 24, 48 and 72 h) and recovery (t = 2 and 24 h). At designated times, 200- $\mu$ l postbranchial haemolymph samples (designated a-arterial) were removed and used to measure pH and total carbon dioxide (C<sub>CO<sub>2</sub></sub>). The remainder of each sample was frozen and subsequently used to determine concentrations of Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Cl<sup>-</sup> and phosphate.

Series 2 reports branchial net fluxes of acidic equivalents and major haemolymph electrolytes. The crayfish in this series were surgically prepared for the collection of urinary outflow as described by Wheatly & Toop (1989), thereby allowing simultaneous measurement of urinary effluxes. The net branchial fluxes were recorded over approximately 13-h flux periods throughout the entire experiment. Water pH varied on average by 0.2 pH units during this time and adjustment was considered unnecessary. Corresponding changes in titration alkalinity were less than 1 mequivl<sup>-1</sup>. A 20-ml water sample was removed at the start and end of each period and used immediately to measure titratable alkalinity; the remainder was frozen for subsequent determination of concentrations of ammonia, Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> and Cl<sup>-</sup>.

The Na<sup>+</sup> and Cl<sup>-</sup> net fluxes were resolved into unidirectional influx and efflux components at times of peak interest which were the transitions from steady-state

normoxia to hyperoxia and from steady-state hyperoxia to normoxia, resembling the protocol employed by Wood *et al.* (1984). Immediately after the flush at the end of the first 24-h normoxic acclimation period, radiotracers were added to the bath water (0.4  $\mu\text{Ci}$  of  $^{22}\text{Na}$  and 0.5  $\mu\text{Ci}$  of  $^{36}\text{Cl}$  in 1 ml of bath water; New England Nuclear) and allowed to mix for 15 min. Two 3-ml water samples were then drawn over three successive 2-h intervals and used to determine  $\gamma$  and  $\beta$  radioactivity (see below). This procedure was repeated for five consecutive 2-h intervals immediately following the switch to hyperoxia, three periods of steady-state hyperoxia ( $t = 51\text{--}57$  h) and the first 10 h of recovery normoxia. Each additional dose of isotope was doubled to improve the external to internal specific activity ratio (see below) considering previous radiotracer loading of the crayfish.

#### *Analytical procedures*

Postbranchial haemolymph pH was determined on a 50- $\mu\text{l}$  subsample using an IL 20985 liquid junction capillary electrode attached to a 213 blood gas analyser.  $\text{CaCO}_2$  (40  $\mu\text{l}$ ) was determined using the Capnicon (Cameron Instruments Inc.). Haemolymph inorganic cation concentrations were measured on thawed, appropriately diluted haemolymph samples using atomic absorption spectrophotometry (see Wheatly & McMahan, 1982; Perkin Elmer model 5000).  $[\text{Cl}^-]$  was determined on a 20- $\mu\text{l}$  subsample of undiluted haemolymph by coulometric titration (Radiometer CMT 10) and total inorganic phosphate on a fourfold dilution using a micromodification of the method described by Atkinson *et al.* (1973).

Water electrolyte concentrations were determined using similar techniques. The  $\text{Cl}^-$  titrator was calibrated to measure concentrations of 0–1 mequiv  $\text{l}^{-1}$  using a 1-ml sample size for standards and unknowns. Water titration alkalinity was determined by titrating an air-equilibrated 10 ml sample to pH 4.00 with 0.02 mol  $\text{l}^{-1}$  HCl (McDonald & Wood, 1981) and ammonia concentration  $[\text{NH}_3 + \text{NH}_4^+]$  was determined in a 5-ml water sample using the phenolphthorite method (Solorzáno, 1969). This technique does not distinguish between the ionized and unionized forms of ammonia; however, given a pK of around 9, the predominant form would be  $\text{NH}_4^+$ .

For the determination of  $\beta$  emissions, one of the water samples was diluted with 6 ml of fluor (ScintiVerse E; Fisher) and counted on a scintillation counter (Beckman model LS 5801); the second sample was counted on a gamma counter (Beckman 4000). Since  $^{22}\text{Na}$  is a mixed  $\gamma$  and  $\beta$  emitter, a set of  $^{22}\text{Na}$  standards was counted on both machines to determine the difference in counting efficiency. This enabled the scintillation counts due to  $^{22}\text{Na}$  to be computed and subtracted from the total, leaving counts due to  $^{36}\text{Cl}$ , which is a pure  $\beta$  emitter. This method has previously been used by Wood *et al.* (1984) and Wood & Rogano (1986).

#### *Calculations*

Postbranchial haemolymph bicarbonate concentration (effectively  $[\text{HCO}_3^- + \text{CO}_3^{2-}]_a$ ) and carbon dioxide tension ( $\text{Pa}_{\text{CO}_2}$ ) were calculated using the Henderson–Hasselbalch equation and values of pK $_1$  and  $\alpha\text{CO}_2$  of 6.119 and

0.0561 mmol l<sup>-1</sup> mmHg<sup>-1</sup>, respectively, which have been determined for this species previously by Wheatly & McMahon (1982). In the acid–base analysis, the haemolymph metabolic acid load ( $\Delta H^+m$ ) was calculated according to McDonald *et al.* (1980) as:

$$\Delta H^+m = [\text{HCO}_3^- + \text{CO}_3^{2-}]_1 - [\text{HCO}_3^- + \text{CO}_3^{2-}]_2 - \beta(\text{pH}_1 - \text{pH}_2), \quad (1)$$

where 1 and 2 refer to successive sampling times and  $\beta$  is  $-11 \text{ mequiv l}^{-1} \text{ pH unit}^{-1}$ , the average nonbicarbonate buffer value determined previously (Wheatly & McMahon, 1982).

Net branchial flux rates of each electrolyte, X, were calculated in  $\mu\text{equiv kg}^{-1} \text{ h}^{-1}$  as:

$$J_{\text{net}}^X = \frac{([\text{X}]_{\text{wi}} - [\text{X}]_{\text{wf}})V}{tW}, \quad (2)$$

where i and f refer to initial and final water (w) sample concentrations of X ( $\mu\text{equiv ml}^{-1}$ ), V is the flux volume corrected for sampling deficits (ml), t is the elapsed time (h), and W is the mass of the crayfish (kg). Using this formula, a negative value indicates a net loss by the crayfish and *vice versa*.

By reversing the i and f terms in the above equation, the net titratable acidity (TA) flux could be calculated from the titratable alkalinities. The net branchial flux of acidic equivalents ( $J_{\text{net}}^{\text{H}}$ ) was calculated as the sum of the titratable acidity ( $J_{\text{net}}^{\text{TA}}$ ) and ammonia ( $J_{\text{net}}^{\text{Am}}$ ) components (McDonald & Wood, 1981).

For each 2-h period under study, the unidirectional influx (in  $\mu\text{equiv kg}^{-1} \text{ h}^{-1}$ ) was calculated using the equation outlined by Maetz (1956):

$$J_{\text{in}}^X = \frac{(R_{\text{Xi}} - R_{\text{Xf}})_w V}{\text{SA}_{\text{Xw}} t W}, \quad (3)$$

where  $R_{\text{Xi}}$  or  $R_{\text{Xf}}$  are radioactivities of X in initial or final water (w) samples (in counts  $\text{min}^{-1} \text{ ml}^{-1}$ ) and  $\text{SA}_{\text{Xw}}$  is the mean specific activity of isotope X in the water (w) during the flux period (in counts  $\text{min}^{-1} \mu\text{equiv}^{-1}$ ) calculated as the mean  $R_{\text{Xw}}$  divided by  $[\text{X}]_{\text{w}}$ . All other symbols are as defined above. In preliminary experiments haemolymph specific activity determined at the end of each period of isotope usage never exceeded 5% of the  $\text{SA}_{\text{Xw}}$ , making backflux correction unnecessary.

Unidirectional effluxes  $J_{\text{out}}^X$  were calculated by the conservation equation:

$$J_{\text{out}}^X = J_{\text{net}}^X - J_{\text{in}}^X. \quad (4)$$

#### Statistical treatment

Data are expressed throughout as mean  $\pm$  s.e.m. (number of observations). Sample means were tested for homogeneity of variance (*F*-test) and compared with control values (each crayfish serving as its own control) using Student's two-tailed *t*-test (paired variates) with  $P = 0.05$  as the confidence limit. Relationships between parameters were identified using standard regression and correlation analyses.

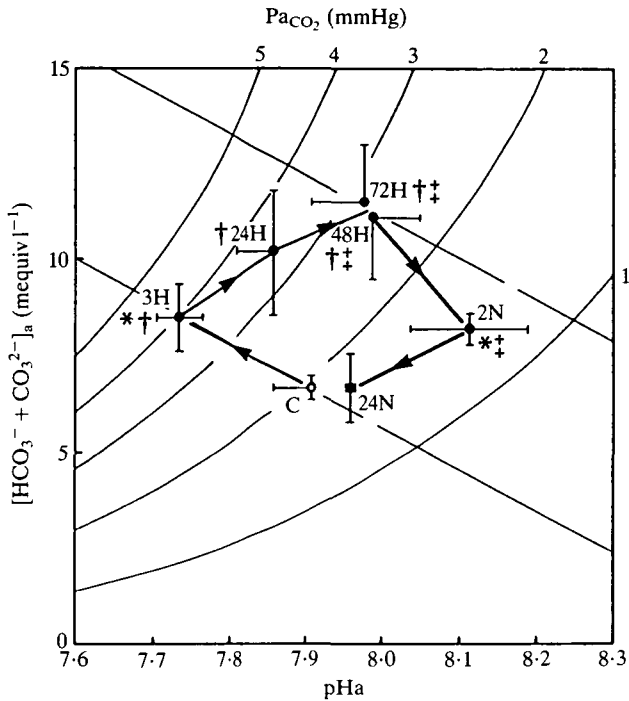


Fig. 1.  $[\text{HCO}_3^- + \text{CO}_3^{2-}]_a$  versus pHa diagram for postbranchial haemolymph of *Pacifastacus leniusculus* (series 1) at  $12^\circ\text{C}$  during control normoxia (C), hyperoxia (H) and recovery normoxia (N). Numbers indicate time in hours for the various treatments.  $\text{PaCO}_2$  was calculated using the Henderson-Hasselbalch equation using  $\text{pK}'_1$  and  $\alpha\text{CO}_2$  values of  $6.119$  and  $0.0561 \text{ mmol l}^{-1} \text{ mmHg}^{-1}$ , respectively. Values are represented as mean  $\pm$  S.E.M. ( $N = 8$ ). The nonbicarbonate buffer line (diagonal) and  $\text{CO}_2$  isopleths were constructed using information obtained previously for this species (Wheatly & McMahon, 1982) and assuming that nonbicarbonate buffering remained constant during the experiment. Symbols denote significance compared to control for pH (\*),  $\text{PCO}_2$  (†) or  $[\text{HCO}_3^- + \text{CO}_3^{2-}]$  (‡).

## Results

### *Haemolymph acid-base and electrolyte status*

Within 3 h of exposure to hyperoxia, pHa fell significantly from  $7.91 \pm 0.05$  (8) to  $7.73 \pm 0.03$  (8) due to a significant increase in  $\text{PaCO}_2$  from  $1.9 \pm 0.2$  (8) to  $4.2 \pm 0.5$  (8) mmHg constituting a classical respiratory acidosis (Fig. 1). Although  $\text{PaCO}_2$  remained significantly elevated throughout hyperoxia, levels partially recovered to around  $2.8$  mmHg. However, pHa progressively returned to control levels at around 36 h owing to an increase in  $[\text{HCO}_3^- + \text{CO}_3^{2-}]_a$  from  $8.6 \pm 0.9$  (8) to  $11.1 \pm 1.6$  mequiv  $\text{l}^{-1}$  which became significant at 48 h. Titration up the  $4$  mmHg  $\text{PCO}_2$  isopleth would indicate that  $[\text{HCO}_3^- + \text{CO}_3^{2-}]_a$  levels had reached  $12.4$  mequiv  $\text{l}^{-1}$  prior to the partial recovery of the respiratory acidosis. Acid-base determinants did not change subsequent to the 48 h sample. The steady-state hyperoxic acid-base status was rapidly reversed upon re-establishment of nor-

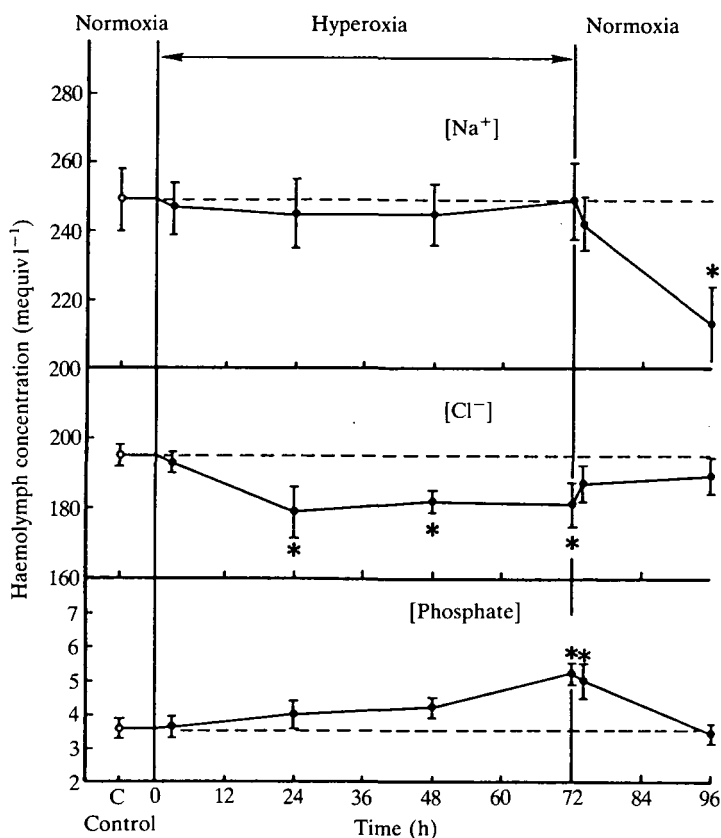


Fig. 2. Time-dependent changes in postbranchial haemolymph concentrations of  $\text{Na}^+$ ,  $\text{Cl}^-$  and phosphate in *Pacifastacus leniusculus* (series 1) during control normoxia (C), hyperoxia and recovery at  $12^\circ\text{C}$ . Consult legend to Fig. 1 for other details. Asterisks denote significant differences from control values.

moxia (Fig. 1).  $\text{PaCO}_2$  recovered completely within 2 h, accounting for  $2 \text{ mequiv l}^{-1}$  of the reduction in  $[\text{HCO}_3^- + \text{CO}_3^{2-}]_a$ , an additional  $1 \text{ mequiv l}^{-1}$  being lost by nonrespiratory means. Circulating  $[\text{HCO}_3^- + \text{CO}_3^{2-}]_a$  remained above control levels producing a significant alkalosis at this time. Both parameters recovered within 24 h.

Circulating  $[\text{Cl}^-]$  decreased significantly within 24 h (Fig. 2) from  $195 \pm 3$  (8) to  $179 \pm 7$  (8)  $\text{mequiv l}^{-1}$  and remained low, but  $[\text{Na}^+]$  remained unchanged.  $[\text{Phosphate}]$  rose progressively to reach levels which were 50% above control by 72 h.  $[\text{Cl}^-]$  and  $[\text{phosphate}]$  recovered within 24 h whereas  $[\text{Na}^+]$  dropped significantly from  $250 \pm 9$  (8) to  $214 \pm 12$  (8)  $\text{mequiv l}^{-1}$ . Control levels of haemolymph  $\text{K}^+$  [ $2.02 \pm 0.14$  (8)  $\text{mequiv l}^{-1}$ ],  $\text{Ca}^{2+}$  [ $17.3 \pm 1.1$  (8)  $\text{mequiv l}^{-1}$ ] and  $\text{Mg}^{2+}$  [ $2.57 \pm 0.18$  (8)  $\text{mequiv l}^{-1}$ ] were unchanged throughout the experiment.

#### Branchial net fluxes of acidic equivalents and ions

Under control conditions a  $J_{\text{net}}^{\text{Amm}}$  of  $-65.4 \pm 12.3$  (8)  $\mu\text{equiv kg}^{-1} \text{h}^{-1}$  was

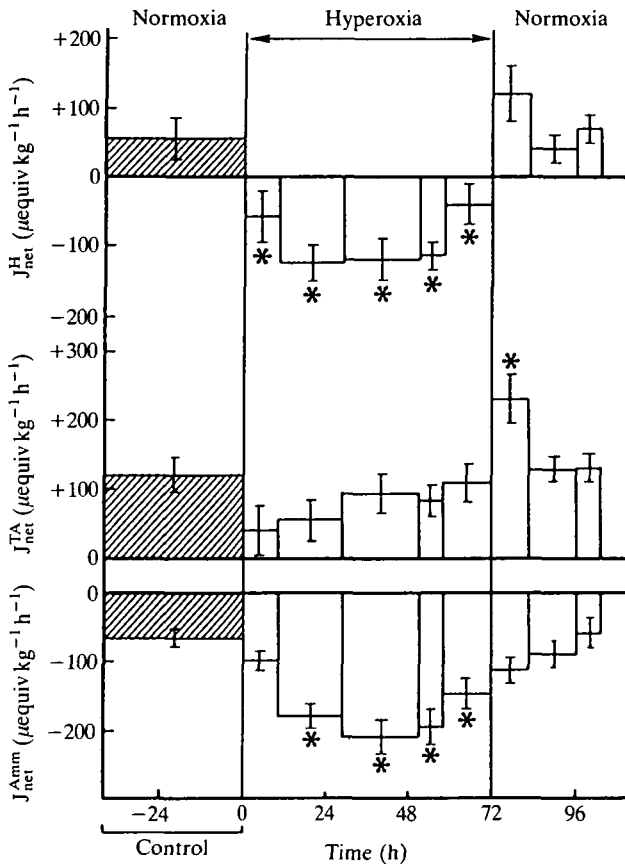


Fig. 3. Time-dependent changes in branchial flux rates of net acidic equivalents ( $J_{\text{net}}^{\text{H}}$ ), titratable acidity ( $J_{\text{net}}^{\text{TA}}$ ) and ammonia ( $J_{\text{net}}^{\text{Amm}}$ ) during control normoxia (values combined for all flux periods from  $t = -48$  h to  $t = 0$  h), hyperoxia and recovery in *Pacifastacus leniusculus* at  $12^{\circ}\text{C}$  (series 2).  $J_{\text{net}}^{\text{H}} = J_{\text{net}}^{\text{TA}} + J_{\text{net}}^{\text{Amm}}$ . By convention, a negative value signifies loss from the crayfish. Consult legends to Figs 1 and 2 for other details.

countered by a  $J_{\text{net}}^{\text{TA}}$  of  $+121.9 \pm 24.6$  (8)  $\mu\text{equiv kg}^{-1} \text{h}^{-1}$  producing a small net uptake of acidic equivalents (Fig. 3). During hyperoxia  $J_{\text{net}}^{\text{Amm}}$  became increasingly negative, reaching levels that were 3 times control within 24 h and that remained significantly elevated throughout hyperoxia.  $J_{\text{net}}^{\text{TA}}$  exhibited a tendency to become reduced during initial hyperoxia. The net result was a negative  $J_{\text{net}}^{\text{H}}$ , in other words an excretion of  $\text{H}^+$  at rates of around  $-100 \mu\text{equiv kg}^{-1} \text{h}^{-1}$  throughout hyperoxia. Immediately upon recovery  $J_{\text{net}}^{\text{Amm}}$  returned to resting whereas  $J_{\text{net}}^{\text{TA}}$  increased significantly, the net effect being a reversal of  $J_{\text{net}}^{\text{H}}$  to positive values, as seen in prehyperoxic controls.

Under control conditions crayfish were losing the major electrolytes  $\text{Na}^+$  and  $\text{Cl}^-$  at the relatively low rates of  $-65 \pm 27$  (8) and  $-132 \pm 40$  (8)  $\mu\text{equiv kg}^{-1} \text{h}^{-1}$ , respectively (Fig. 4). During the first 24 h of hyperoxia  $J_{\text{net}}^{\text{Cl}}$  doubled to around  $-250 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ , subsequently returning to the control level;  $J_{\text{net}}^{\text{Na}}$ , mean-



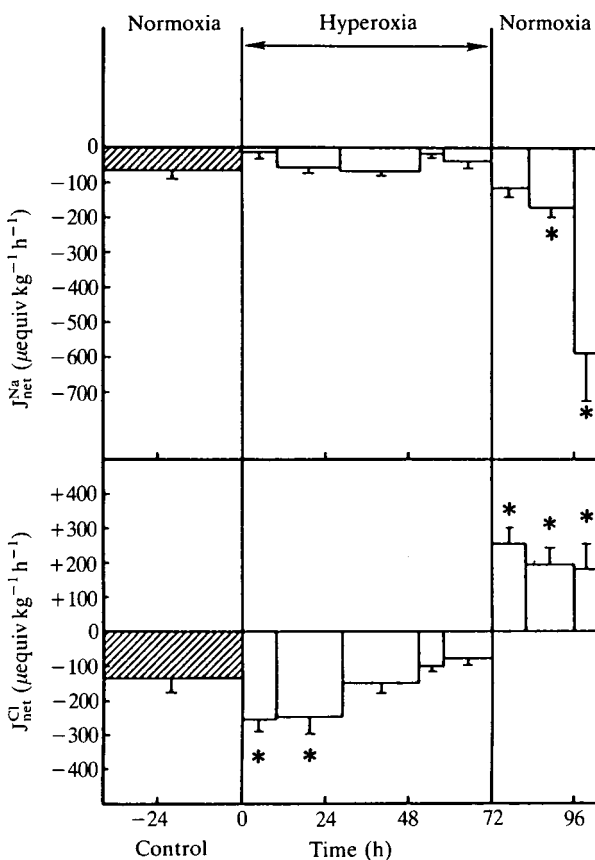


Fig. 4. Time-dependent changes in branchial net flux rates of sodium ( $J_{\text{net}}^{\text{Na}}$ ) and chloride ( $J_{\text{net}}^{\text{Cl}}$ ) during control normoxia, hyperoxia and recovery in *Pacifastacus leniusculus* at 12°C. Consult legends to Figs 1 and 3 for additional information.

while, was unchanged. Upon recovery significant changes were observed in both parameters.  $J_{\text{net}}^{\text{Cl}}$  changed immediately to a net uptake of  $+200 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ .  $J_{\text{net}}^{\text{Na}}$  became progressively negative, reaching levels of  $-600 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ . Neither flux had re-established equilibrium after 31 h.

Under normoxic conditions crayfish were also losing  $\text{Ca}^{2+}$  branchially at rates approaching  $-40 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ , but were essentially in ion balance with respect to  $\text{Mg}^{2+}$  and  $\text{K}^{+}$  (Fig. 5).  $J_{\text{net}}^{\text{Ca}}$  exhibited a tendency to become increasingly negative during the later period of hyperoxic exposure, a pattern which was repeated during recovery.  $\text{Mg}^{2+}$  was lost at initial rates of  $-30 \mu\text{equiv kg}^{-1} \text{h}^{-1}$  which progressively recovered within 48 h.  $J_{\text{net}}^{\text{Mg}}$  was not significantly changed during recovery. Crayfish entered negative  $\text{K}^{+}$  balance in the later stages of hyperoxic exposure, with  $J_{\text{net}}^{\text{K}}$  values approaching  $-10 \mu\text{equiv kg}^{-1} \text{h}^{-1}$  which persisted throughout recovery.

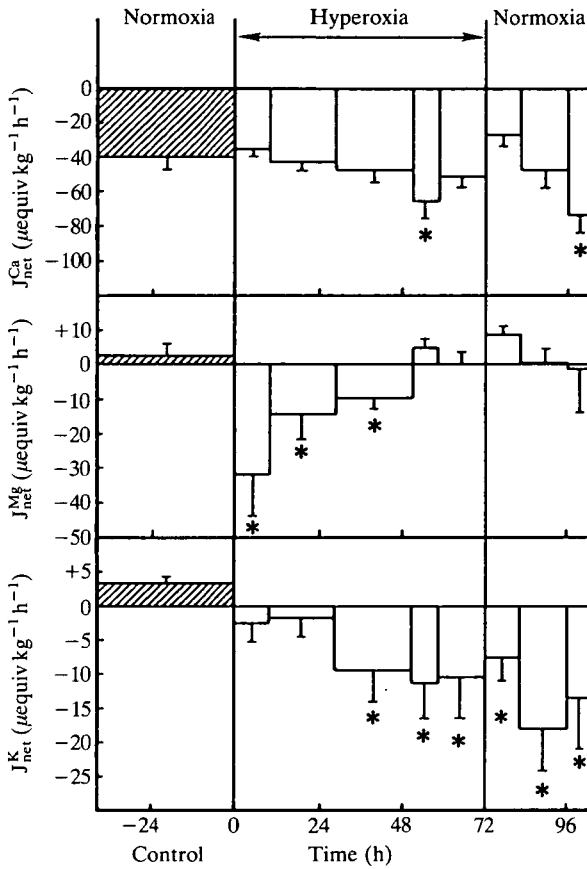


Fig. 5. Time-dependent changes in branchial net flux rates of calcium ( $J_{net}^{Ca}$ ), magnesium ( $J_{net}^{Mg}$ ) and potassium ( $J_{net}^{K}$ ) during control normoxia, hyperoxia and recovery in *Pacifastacus leniusculus* (series 2) at 12°C. Consult legends to Figs 1 and 3 for additional information.

#### Branchial unidirectional $Na^+$ and $Cl^-$ fluxes

In control crayfish a  $J_{in}^{Na}$  of  $+263.4 \pm 15.8$  (8)  $\mu\text{equiv kg}^{-1} \text{h}^{-1}$  was countered by a  $J_{out}^{Na}$  of  $-346.9 \pm 19.5$   $\mu\text{equiv kg}^{-1} \text{h}^{-1}$  (Fig. 6). During the initial 8 h of hyperoxic exposure, significant and parallel reductions in both components were measured, with fluxes in each case dropping to around 50% of their control values. Steady-state hyperoxic values were similar to control and remained unchanged during recovery.

Control unidirectional fluxes of  $Cl^-$  (Fig. 6) were larger than corresponding  $Na^+$  fluxes by 16% for influx [ $+306.3 \pm 48.9$  (8)  $\mu\text{equiv kg}^{-1} \text{h}^{-1}$ ] and 35% for efflux [ $-467.9 \pm 57.3$  (8)  $\mu\text{equiv kg}^{-1} \text{h}^{-1}$ ]. During initial hyperoxic exposure  $J_{in}^{Cl}$  became significantly reduced from  $+300$   $\mu\text{equiv kg}^{-1} \text{h}^{-1}$  to around  $+100$   $\mu\text{equiv kg}^{-1} \text{h}^{-1}$ . Control levels were re-established during the later stages of hyperoxia. During recovery  $J_{in}^{Cl}$  doubled for the initial 4 h, thereafter returning to

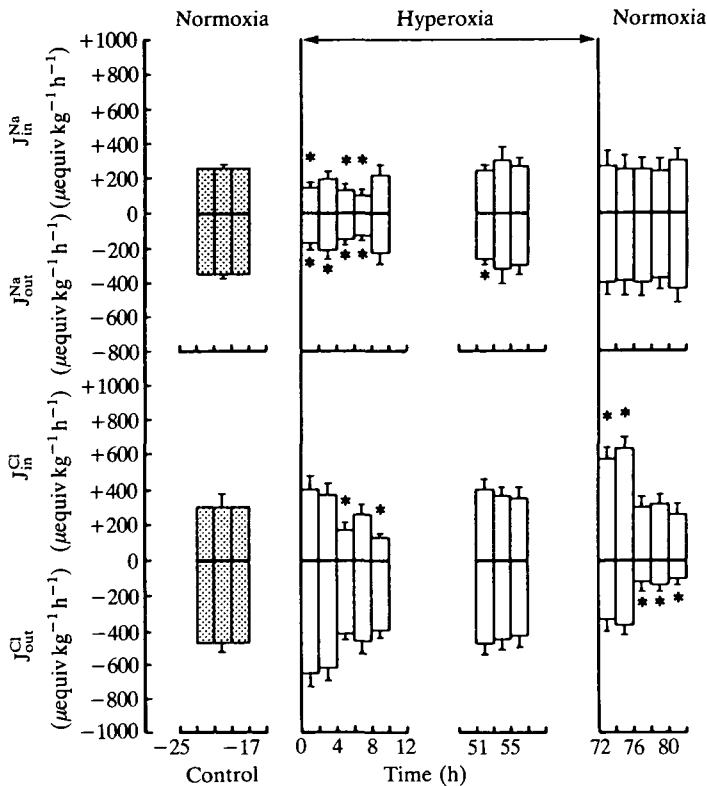


Fig. 6. Branchial unidirectional influxes and effluxes of sodium ( $J_{in}^{Na}$ ,  $J_{out}^{Na}$ ) and chloride ( $J_{in}^{Cl}$ ,  $J_{out}^{Cl}$ ) during control normoxia,  $t = 0-10$  h and 51–57 h of hyperoxia and  $t = 0-10$  h of recovery normoxia in *Pacifastacus leniusculus* (series 2) at 12°C. The three normoxic control periods were combined to give a mean value against which statistical significance of each flux period has been tested. As before, positive values signify movement into the crayfish and *vice versa*.

resting.  $J_{out}^{Cl}$ , meanwhile, exhibited a significant reduction to 20% of control values.

## Discussion

### *Haemolymph acid–base and electrolyte status*

Control postbranchial acid–base parameters in *Pacifastacus* were typical of values reported previously in crayfish (Dejours & Armand, 1980; Wheatly & Taylor, 1981; Wilkes & McMahon, 1982; Wood & Rogano, 1986). Fish exhibit more pronounced acid–base disturbances when exposed to identical hyperoxic levels (Höbe *et al.* 1984). Branchial vasoconstriction in this case may explain the reduced branchial gas transfer factor (Wilkes *et al.* 1981). In decapods the diffusional characteristics are less affected (M. G. Wheatly, in preparation, in

*Cancer*) which may be related to the lack of vascular smooth muscle in the circulatory system. The time course for metabolic compensation observed in the crayfish in this study was far slower than previously reported in marine crabs (Truchot, 1975; Wheatly, 1987), perhaps because the electroneutral ion exchanges are limited by  $\text{Na}^+$  and  $\text{Cl}^-$  levels in fresh water (Evans, 1986).

A similar partial recovery of  $\text{PaCO}_2$  with time has been observed during long-term hyperoxia in the marine crab *Cancer* (Wheatly, 1987) but not in fish (Wilkes *et al.* 1981; Hōbe *et al.* 1984). In *Cancer* an initial hypoventilation and bradycardia progressively recovered, perhaps in an attempt to avoid low gas transfer efficiencies associated with chronic hypoperfusion (Burggren *et al.* 1974) and hypoventilation (Burggren & McMahon, 1983).

The reduction in circulating  $[\text{Cl}^-]$  which accompanied the increase in  $[\text{HCO}_3^- + \text{CO}_3^{2-}]$  may be the most direct evidence for acid-base regulation *via* ion exchange. The mechanism involved, however, is not a simple 1:1 exchanger since the two parameters did not change stoichiometrically and the net charge balance revealed a net loss of anions.

### *Branchial exchange of acidic equivalents and ions*

#### *Control values*

It is difficult to compare the present branchial ion and acid fluxes with previous studies (e.g. Shaw, 1959, 1960*a,b*; Ehrenfeld, 1974; Wood & Rogano, 1986) because of disparate methodologies for example, using ionically depleted animals, using experimental media of different ionic composition, or failure to separate renal responses from whole-body exchange. In particular there is considerable variation between relative magnitudes of unidirectional  $\text{Na}^+$  and  $\text{Cl}^-$  fluxes which would appear to reflect the  $[\text{Na}^+]$ ,  $[\text{Cl}^-]$  and  $[\text{Ca}^{2+}]$  of the external medium. Shaw's (1960*a,b*) nonlinear relationship between influx and external concentration would predict comparable rates for  $J_{\text{in}}^{\text{Na}}$  and  $J_{\text{in}}^{\text{Cl}}$  at the external levels used in this study. The external NaCl concentration employed by Wood & Rogano (1986) is on the steeply rising phase of this relationship, explaining why their control  $J_{\text{in}}^{\text{Cl}}$  values were three times greater than  $J_{\text{in}}^{\text{Na}}$ . Water  $[\text{Ca}^{2+}]$  may also be a contributory factor since reducing  $[\text{Ca}^{2+}]$  reproduced this discrepancy (M. G. Wheatly, unpublished observation).

#### *Experimental values*

The extracellular accumulation of metabolic base (Fig. 7) during the initial 48 h of hyperoxia was accompanied by branchial acid excretion (Fig. 3). Upon recovery, rapid removal of the base load from the haemolymph was associated with branchial base excretion. Transbranchial exchange would therefore appear to be a major avenue for acid-base regulation, at least on a qualitative basis. Constructing an acidic equivalent budget, however, reveals some interesting deficits (Table 1). Only 17% of the total base accumulated during hyperoxia was buffered in the extracellular fluid, suggesting that the majority of  $\text{H}^+$  lost to the

## Hyperoxia in crayfish – acid and ion exchange

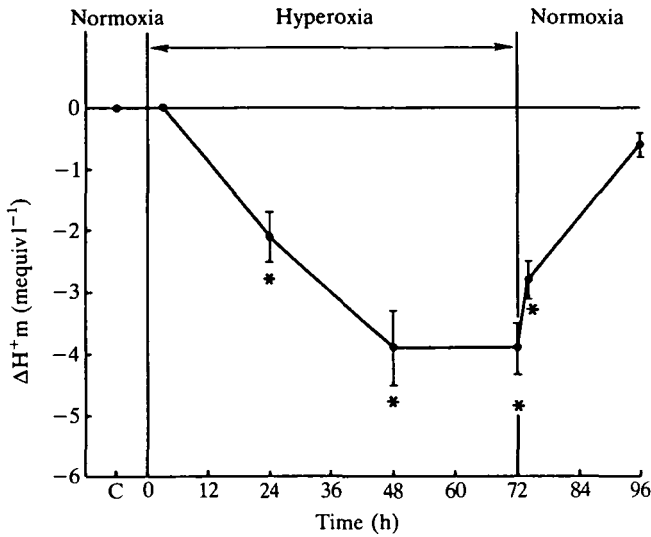


Fig. 7. The cumulative metabolic acid load in the haemolymph of *Pacifastacus leniusculus* (series 1) during control normoxia (C), hyperoxia and recovery at 12°C (calculated from information contained in Fig. 1). Consult legend to Fig. 1 for other details.

experimental water originated from another fluid compartment, presumably intracellular fluid or carapace, agreeing with another study on exposure of crayfish to acid soft water (Wood & Rogano, 1986). During recovery, 41 % of the  $H^+$  load was attributed to extracellular changes. More importantly, although 85 % of the change in  $[H^+]$  in the extracellular fluid was corrected within 24 h of recovery, the branchial  $H^+$  uptake was only half that predicted based on the measured net  $H^+$  excretion during 72 h of hyperoxia (2308 vs 6726  $\mu\text{equiv kg}^{-1}$ ).

Accumulation of metabolic  $[HCO_3^- + CO_3^{2-}]$  was also accompanied by significant changes in net branchial electrolyte fluxes (Figs 4,5). Increased branchial  $Cl^-$  loss (Fig. 4) over the initial 24 h of hyperoxia paralleled the time course of extracellular  $[HCO_3^- + CO_3^{2-}]$  accumulation and no doubt explained the reduction in circulating levels of  $Cl^-$  (Fig. 2). Complementary changes in  $J_{net}^{Na}$  were not apparent (Fig. 4) which was consistent with maintained haemolymph levels (Fig. 2). Trout exhibited similar large negative  $J_{net}^{Cl}$  during hyperoxia (Wood *et al.* 1984) with less pronounced changes in  $J_{net}^{Na}$ . Furthermore, in both studies, the return to normoxia had a far greater stimulatory effect on both  $Na^+$  and  $Cl^-$  transport, with  $J_{net}^{Na}$  becoming increasingly negative and  $J_{net}^{Cl}$  increasingly positive. Essentially the reverse trends were seen during metabolic acidosis induced by environmental acidity in *Orconectes propinquus* (Wood & Rogano, 1986).

Extrarenal  $Ca^{2+}$  loss (Fig. 5) can be tolerated with no ill effect in intermoult crayfish, thereby reducing the energy required for active uptake (Greenaway, 1972). This net efflux, which presumably represents diffusional loss, was essentially unchanged during hyperoxia. The progressive net branchial  $K^+$  loss (Fig. 5)

Table 1. A comparison of net fluxes with the environmental water and changes in the extracellular pool during 72 h of hyperoxia and 24 h of recovery in *Pacifastacus leniusculus*

Ion	Change in concentration ( $\mu\text{equiv kg}^{-1}$ crayfish)					
	72 h hyperoxia			24 h recovery		
	Experimental water	Extracellular space	Intracellular space/carapace	Experimental water	Extracellular space	Intracellular space/carapace
H <sup>+</sup>	-6726	-1139	-5587	+2308	+964	+1344
Na <sup>+</sup>	+1242	0	+1242	-1905*	0	-1905
Cl <sup>-</sup>	-2817	-3854	+1037	+10471	+2102	+8369
PO <sub>4</sub> <sup>2-</sup>	ND	+444	ND	ND	-584	ND
Ca <sup>2+</sup>	-564	0	-564	-217	0	-217
Mg <sup>2+</sup>	-932	0	-932	+9	0	+9
K <sup>+</sup>	-718	0	-718	-521	0	-521
Net change	-4881	+2271	-7596	-10797	-554	-9659

Extracellular space was calculated assuming an extracellular fluid volume of 292 ml kg<sup>-1</sup> (see Wheatly & Toop, 1989).

ND, not measured in present experiment.

\* 24 h recovery values not incorporated into this estimate owing to large unexplained Na<sup>+</sup> losses.

must originate intracellularly since circulating levels were unchanged. Transmembrane ion exchanges are reported in a separate paper (M. G. Wheatly & E. C. Vevera, in preparation). To my knowledge these are the first reported data for extrarenal  $Mg^{2+}$  fluxes in crayfish (Fig. 5). Circulating levels would suggest active uptake of  $Mg^{2+}$ , although the sites and mechanisms as well as the observed changes during hyperoxia remain unexplained.

Significant changes in unidirectional fluxes of both  $Na^+$  and  $Cl^-$  (Fig. 6) accounted for the changes in net fluxes (Fig. 4). Parallel reductions in both  $J_{in}^{Na}$  and  $J_{out}^{Na}$  during the initial period of hyperoxia can best be explained as a reduction in the exchange diffusion component (i.e.  $Na^+/Na^+$ ). In trout (Wood *et al.* 1984) recovery from hyperoxia was marked by a significant reduction in  $J_{in}^{Na}$  and increase in  $J_{out}^{Na}$ . During initial exposure to acid in *Orconectes* (Wood & Rogano, 1986) there was a 50% inhibition of  $J_{in}^{Na}$  at unchanged  $J_{out}^{Na}$ , which was explained as  $H^+$  vs  $Na^+$  competition for a common carrier. During long-term metabolic acidosis, however, Wood & Rogano (1986) reported an increase in  $Na^+$  exchange diffusion.

A reduction in  $J_{in}^{Cl}$  (Fig. 6) explains the net branchial loss during initial hyperoxic exposure (Fig. 4) which was similar to that in trout. During recovery the reverse sequence of events occurred with, in addition, a significant reduction in  $J_{out}^{Cl}$ . In trout both components increased, particularly  $J_{in}^{Cl}$ , explaining the net  $Cl^-$  uptake. As in hyperoxia,  $J_{out}^{Cl}$  was less affected during metabolic acidosis in crayfish (Wood & Rogano, 1986). A maintained 30% inhibition of  $J_{in}^{Cl}$  was interpreted as conformational changes in the carrier and/or reduction of internal  $HCO_3^-$  levels potentially impeding  $Cl^-/HCO_3^-$  exchange. Cameron (1978) demonstrated an increased  $Na^+$  relative to  $Cl^-$  influx during hypercapnia in blue crabs but questioned the validity of his measurements because of high background concentrations and baseline branchial flux rates in a marine species.

The charge budget during hyperoxia (Table 1) reveals a net charge imbalance in the experimental water equal to 73% of the  $J_{net}^H$  (where net charge =  $H^+ + Na^+ + K^+ + Ca^{2+} + Mg^{2+} - Cl^- - PO_4^{2-}$ ) which, for the purpose of the present investigation, must once again be attributed to the intracellular fluid compartment. The analysis would suggest that during hyperoxia there is a substantial efflux of  $H^+$  from the tissues accompanied by an uptake of  $Na^+$  in excess of  $Cl^-$ , with reverse trends on recovery. We have recently confirmed these trends experimentally (M. G. Wheatly & E. C. Vevera, in preparation). Wood & Rogano (1986) also found that net charge was best balanced in the haemolymph of the three compartments studied. The majority of whole-body  $H^+$ ,  $K^+$  and  $Ca^{2+}$  exchange originated outside the extracellular fluid and  $Cl^-$  fluxes in the tissues and haemolymph were exactly balanced. This suggests that  $Cl^-$  removal from the blood during hyperoxia may be partly by intracellular translocation.

#### *Relationships between branchial ion and acid–base exchange*

Correlations between branchial acidic equivalent (Fig. 3) and electrolyte fluxes (Figs 4, 5, 6) were identified in the same way as by Wood *et al.* (1984) in trout and by Wood & Boutilier (1985) in a land crab. For flux components determined

Table 2. Correlation between  $J_{net}^H$  (independent variable) and various branchial electrolyte flux components (dependent variables listed in left column) in *Pacifastacus leniusculus* (series 2) at 12°C

	Dependent variable ( $\mu\text{equiv kg}^{-1} \text{h}^{-1}$ )	Intercept	Slope	Correlation coefficient ( $N$ )
(1)	$J_{net}^{Na} + J_{net}^K + J_{net}^{Ca}$ $+ J_{net}^{Mg} - J_{net}^{Cl}$	-194.3	-2.55	-0.70 (72)
(2)	$J_{net}^{Na}$	-143.9	-0.99	-0.50 (72)
(3)	$J_{net}^{Cl}$	-8.8	+1.64	0.80 (72)
(4)	$J_{net}^{Na} - J_{net}^{Cl}$	-135.3	-2.60	-0.73 (72)
(5)	$J_{in}^{Na}$	239.0	0.14	0.27 (32)
(6)	$J_{out}^{Na}$	-305.5	-0.65	-0.73 (32)
(7)	$J_{in}^{Cl}$	340.0	1.09	0.64 (32)
(8)	$J_{out}^{Cl}$	-420.0	1.44	0.70 (32)
(9)	$J_{in}^{Na} - J_{in}^{Cl}$	-100.0	0.95	-0.69 (32)
(10)	$J_{out}^{Na} - J_{out}^{Cl}$	116.4	-2.10	-0.72 (32)

throughout the entire experiment (e.g.  $J_{net}^{Na}$ ,  $J_{net}^{Cl}$ ) or calculated from them (e.g.  $J_{net}^{Na} - J_{net}^{Cl}$ ) there were nine separate flux periods. The complete data set (72 data points for eight crayfish) was subjected to regression and correlation analysis with  $J_{net}^H$  as the independent variable (Table 2). For the components measured using radiotracers (e.g.  $J_{in}^{Na}$ ,  $J_{out}^{Cl}$ , etc.) fluxes were averaged over each of the four periods of isotope use and regressed against the corresponding  $J_{net}^H$ , producing a smaller data set ( $N = 32$ ).

In terms of simple chemistry,  $J_{net}^H$  should equal the difference between measured strong cation and anion fluxes (i.e.  $J_{net}^{Na} + J_{net}^K + J_{net}^{Ca} + J_{net}^{Mg} - J_{net}^{Cl}$ ) ideally with a slope of 1 and intercept passing through the origin (see Wood & Boutilier, 1985). Since the data do not fulfil these criteria [Table 2 (1)], it would appear that other unmeasured ions are involved. The relative contributions of the two major electrolytes for which unidirectional fluxes were available can be evaluated. As expected,  $J_{net}^{Na}$  [Table 2 (2)] was correlated negatively, and  $J_{net}^{Cl}$  [Table 2 (3)] positively, with  $J_{net}^H$ . Of the two,  $J_{net}^{Cl}$  had the stronger coefficient and a greater slope, suggesting that it was more important in determining  $J_{net}^H$ . Furthermore, the intercept was virtually zero, suggesting that there was no net  $Cl^-$  output in the absence of acidic equivalent output. In this analysis  $J_{net}^H$  showed no better correlation with  $J_{net}^{Na} - J_{net}^{Cl}$  [Table 2 (4)] than with the difference between measured strong cations and anions [Table 2 (1)], again implicating the involvement of unmeasured electrolytes. In previous studies these indices have completely explained measured  $J_{net}^H$  (Wood *et al.* 1984; Wood & Boutilier, 1985).

Extending the analysis to unidirectional fluxes revealed high positive correlations between  $J_{net}^H$  and both  $Cl^-$  influx [Table 2 (7)] and  $Cl^-$  efflux [Table 2 (8)] components. The slope in each case approached unity. The intercepts for both were significantly different from 0 and were approximately equivalent, indicating



that around  $380 \mu\text{equiv kg}^{-1} \text{h}^{-1}$  of  $\text{Cl}^-$  exchange is *via* exchange diffusion ( $\text{Cl}^-/\text{Cl}^-$ ). A corresponding value for  $\text{Na}^+/\text{Na}^+$  exchange diffusion would be around  $270 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ . These values are approximately double those found in the trout (Wood *et al.* 1984) and can account for virtually all of the unidirectional ion fluxes measured in control crayfish (see Fig. 6). Differences between influxes [Table 2 (9)] and effluxes [Table 2 (10)] both provided good correlation with  $J_{\text{net}}^{\text{H}}$  and the slope approached 1 for  $J_{\text{in}}^{\text{Na}} - J_{\text{in}}^{\text{Cl}}$ .

Performing an identical exercise on Wood & Rogano's (1986) crayfish flux data produces a similar conclusion: namely that  $J_{\text{net}}^{\text{H}}$  cannot be entirely explained in terms of differences in net fluxes of strong cations and anions. They did find, however, that  $\text{Na}^+$  and  $\text{Cl}^-$  net fluxes contributed equally to  $J_{\text{net}}^{\text{H}}$ . Collectively these two studies confirm that a large portion of the influx and efflux components of both  $\text{Na}^+$  and  $\text{Cl}^-$  net flux reflects exchange diffusion. This being the case, only a small part of  $\text{Na}^+/\text{Cl}^-$  uptake may represent exchange for acidic/basic equivalents, which may explain why correlations between the two are less easily discernible and (where present) more complex than in rainbow trout (Wood *et al.* 1984).

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